RESEARCH ARTICLE

Cell culturability of *Pseudomonas protegens* CHA0 depends on soil pH

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Abstract

Pseudomonas inoculants may lose colony-forming ability in soil, but soil properties involved are poorly documented. Here, we tested the hypothesis that soil acidity could reduce persistence and cell culturability of *Pseudomonas protegens* CHA0. At 1 week *in vitro*, strain CHA0 was found as culturable cells at pH 7, whereas most cells at pH 4 and all cells at pH 3 were noncultured. In 21 natural soils of contrasted pH, cell culturability loss of *P. protegens* CHA0 took place in all six very acidic soils (pH < 5.0) and in three of five acidic soils (5.0 < pH < 6.5), whereas it was negligible in the neutral and alkaline soils at 2 weeks and 2 months. No correlation was found between total cell counts of *P. protegens* CHA0 and soil composition data, whereas colony counts of the strain correlated with soil pH. Maintenance of cell culturability in soils coincided with a reduction in inoculant cell size. Some of the noncultured CHA0 cells were nutrient responsive in Kogure's viability test, both *in vitro* and in soil. Thus, this shows for the first time that the sole intrinsic soil composition factor triggering cell culturability loss in *P. protegens* CHA0 is soil acidity.

Introduction

Fluorescent *Pseudomonas* strains have been extensively studied for environmental applications in relation to biocontrol of soilborne phytopathogens (Haas & Défago, 2005) and insects (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013), biofertilization of crops (Mirza *et al.*, 2006; Couillerot *et al.*, 2009), and bioremediation of polluted soils (Cycoń *et al.*, 2009). When used as microbial inoculants, these fluorescent pseudomonads may be confronted to adverse conditions in the soil ecosystem, and typically, their cell number tends to decrease after release in the environment (Keel & Défago, 1997; Fischer *et al.*, 2010; Troxler *et al.*, 2012). This is often attributed to biotic interactions, primarily competition (Fliessbach *et al.*, 2009) and predation (Pedersen *et al.*, 2009), but abiotic properties may also play an important part (Sørensen *et al.*, 2001).

Monitoring of the survival of *Pseudomonas* inoculants has often been carried out by colony counts (Fliessbach *et al.*, 2009; Fischer *et al.*, 2010), but the occurrence of cells in a noncultured state limits the significance of colony counts (Pedersen & Leser, 1992; Binnerup *et al.*, 1993; Chapon *et al.*, 2003; Arana *et al.*, 2010; Buck & Oliver, 2010; Troxler *et al.*, 2012). The possibility that at least some of the noncultured bacterial cells are in fact moribund cells has been hypothesized, yet noncultured bacterial cells may remain in this state for months (and perhaps even longer) and some of them respond to nutrient stimulation (Troxler *et al.*, 1997b; Mascher *et al.*, 2002). In some cases, they might even recover cell culturability (Grey & Steck, 2001; Du *et al.*, 2007; Gorshkov *et al.*, 2009; Kana & Mizrahi, 2010). On this basis, it has also been proposed that the noncultured state(s) of cells could represent a persistence strategy of bacteria confronted to stress (Binnerup & Sørensen, 1993; Kragelund & Nybroe, 1994; Troxler *et al.*, 1997b; Oliver, 2010). Regardless of its ecological significance, the environmental factors modulating the ability of bacteria to persist as noncultured cells in soil remain poorly understood and deserve research attention.

Pseudomonas protegens (previously *P. fluorescens*) strain CHA0 has been extensively studied for its biocontrol

properties (Haas & Défago, 2005; Péchy-Tarr *et al.*, 2008; Henkes *et al.*, 2011). Under *in vitro* conditions, this pseudomonad may lose cell culturability when exposed to a combination of oxygen deprivation and reducing conditions (simulating plough pan environments; Mascher *et al.*, 2000), to heavy metal or to acidity (Rezzonico *et al.*, 2003), but not when subjected to hydrogen peroxide (Hase *et al.*, 2000) or nutrient limitation (Hase *et al.*, 1999). The loss of cell culturability under oxygen-deprived/reducing conditions (Mascher *et al.*, 2000) was also observed when the corresponding plough pan conditions were implemented in soil microcosms (Mascher *et al.*, 2003) or investigated in the field (Troxler *et al.*, 2012). However, in the absence of external stress factors (i.e. resulting from field management and/or climatic events) such as plough pan conditions, almost nothing is known on intrinsic soil properties conducive to cell culturability loss in pseudomonads. Noncultured cells of *P. protegens* CHA0 were found when the strain was introduced into acidic bulk soil (Hase *et al.*, 2000), pointing to acidity as a possible factor involved, but it is possible that other soil properties (texture, organic matter quality, bulk density, etc.) could have also played a part in that experiment.

The objective of this work was to assess whether soil acidity was a factor triggering loss of colony-forming ability in *P. protegens* CHA0. To this end, the persistence of the strain was assessed at different pH levels under *in vitro* conditions, as well as in 21 natural soils of contrasted pH, texture and organic matter content. Strain monitoring was carried out using colony counts and direct microscopic cell counts following immunofluorescence staining (Pedersen & Leser, 1992; Heijnen *et al.*, 1995; Hase *et al.*, 2000; Mascher *et al.*, 2003; Troxler *et al.*, 2012). Direct viable counts (DVC) of nutrient-responsive cells were also determined, based on the Kogure's method (Kogure *et al.*, 1979; Troxler *et al.*, 1997b; Mascher *et al.*, 2002), in which nutrient-responsive cells (viable) become larger after treatment with yeast extract (nutrient stimulation) and nalidixic acid (inhibition of cell division), whereas nonresponsive cells remain of small size.

Materials and methods

Strain

Experiments were carried out with strain CHA0-Rif (Natsch *et al.*, 1994), a spontaneous rifampicin-resistant mutant of the biocontrol agent *P. protegens* CHA0 (Stutz *et al.*, 1986; Ramette *et al.*, 2011). Both strains are similar in terms of growth rates and production of the antimicrobial metabolites 2,4-diacetylphloroglucinol and pyoluteorin in laboratory media (Natsch *et al.*, 1994), as well as protection of cucumber from damping-off caused by *Pythium ultimum* (C. Keel, unpublished). Strain CHA0-Rif has been used extensively in ecological assessments (e.g. Natsch *et al.*, 1994; Mascher *et al.*, 2003; Fliessbach *et al.*, 2009; Troxler *et al.*, 2012). For long-term storage of the strain, 1 mL freshly grown bacterial suspension (in King's B medium; King *et al.*, 1954) was mixed with 1 mL glycerol (87%) and conserved at −80 °C.

Acidity experiment *in vitro*

Strain CHA0-Rif was grown overnight at 28 °C with shaking (at 1 ***g***) in liquid King's B medium (King *et al.*, 1954) containing rifampicin (100 μg mL^{-1}; Rif100). The cells were washed two times in sterile distilled water and used to inoculate King's B plates. After a 22-h incubation at 28 °C, the cells were harvested and resuspended in sterile distilled water. The cell suspension was adjusted optically to 10^{10} cells mL^{-1} (confirmed by colony counts) and used to inoculate (at 10^7 cells mL^{-1}) 100-mL Erlenmeyer flasks containing 50 mL of solution of pH 7 (i.e. control; 0.12 mM $Na_2HPO_4{\cdot}2H_2O$ and 0.12 mM $NaH_2PO_4{\cdot}H_2O$), pH 4 (600 mL citric acid 0.1 M and 400 mL Na_2HPO_4 0.2M) or pH 3 (250 mL glycine 0.2 M and 55 mL HCl 0.2 M per litre), as in Rezzonico *et al.* (2003).

The flasks were incubated at 28 °C with shaking (at 1 ***g***). pH values were stable during the 1-week incubation. At 1 week, aliquots were sampled, dilution series were prepared in sterile distilled water, and cell numbers were determined by colony counts, total cell counts and DVC (procedures described below).

Soil experiment

A total of 21 different soil horizons (Supporting Information, Table S1) covering a wide range of pH values (4.3–8.0), soil textures (sand to clay) and organic matter content (0.5–19%; Table S2) were chosen with the help of soil map information from 18 field locations throughout Switzerland (Table S3). Sampling took place in the spring (April). The soils were air-dried until they were friable. Roots and stones were removed, and the soils were sieved through a 5-mm mesh screen. They were dried further to reach a water potential of about −0.03 MPa. Water content at −0.03 MPa (Table S2) was estimated using the filter paper method (McInnes *et al.*, 1994) and confirmed using porous plates (Richard, 1948). Microcosms were set in 25-mL glass vials and consisted of 10 g (wet weight) of natural (i.e. nonsterile) soil.

For inoculation, strain CHA0-Rif was grown overnight at 28 °C with shaking (at 1 ***g***) in liquid King's B medium containing Rif100 until mid-log phase. The cells were

washed twice in sterile distilled water, resuspended in sterile distilled water, and the suspension adjusted optically to about 10^{10} cells mL^{-1} (confirmed by colony counts). Strain CHA0-Rif was added to the microcosms at 10^8 cells g^{-1} dry soil using 80–100 μL cell suspension (amount adapted according to soil dry weight). The 21 controls consisted of uninoculated soil microcosms. The vials were placed in loosely caped 250-cm^3 plastic containers, and the latter were incubated in the dark at 12 °C and 70% relative humidity (Mascher *et al.*, 2000). Water loss in the microcosms was monitored every 3–4 days by weighing the vials, and sterile distilled water was added when necessary to keep water potential at −0.03 MPa.

Destructive sampling was performed at 2 weeks and 2 months, and (for the six very acidic soils KRA, KRB, LAF, LAP, SIG and SIL, the three acidic soils AUW, LAU and ZWE, the neutral soil RIB and the alkaline soil DIE) at 4 months after inoculation. The whole content of each microcosm was extracted by shaking (15 min at 2 ***g***) in sterile distilled water (soil water ratio of 1 : 10 w/v), and dilution series were prepared in sterile distilled water. Cell numbers of *P. protegens* CHA0-Rif were determined by colony counts and total cell counts, as described below.

Cell measurements

The total number of cells of *P. protegens* CHA0-Rif was determined by immunofluorescence microscopy, as described (Troxler *et al.*, 1997b; Mascher *et al.*, 2000), using a primary antiserum specific for strain CHA0 (Troxler *et al.*, 1997a). In the soil experiment, samples from the uninoculated treatments were also studied and no cross-reaction was found. One filter was prepared for each liquid or soil sample, and cells of CHA0-Rif were counted over the entire filter using a Zeiss Axioskop epifluorescence microscope (filters 450–490 nm; at least 20 fields and/or 150 bacterial cells). Detection limit was set at 1 cell in 100 fields, corresponding to 10^5 cells g^{-1} dry soil. Soils were studied at 2 weeks and at 2 months, and cell morphology of CHA0-Rif in the 21 soils at 2 weeks after inoculation was assessed by immunofluorescence microscopy, using 100 randomly chosen cells from each replicate of each soil.

The number of nutrient-responsive cells of strain CHA0-Rif was determined using the technique of Kogure *et al.* (1979), in combination with immunofluorescence microscopy. This was performed in the *in vitro* experiment, as well as at 4 months for 11 selected soils. For the latter, soils were dispersed in 0.77 mM tetrasodium pyrophosphate (Fluka AG, Buchs, Switzerland) to improve cell extraction yield. Pearson's correlation coefficient for colony counts of CHA0-Rif with or without tetrasodium pyrophosphate treatment ($r = 0.86$) was highly significant ($P = 1 \times 10^{-5}$). Nutrients (yeast extract; 250 μg mL^{-1}) and a cell division inhibitor (nalidixic acid; 20 μg mL^{-1}) were then added to the samples, which were incubated for 6 h at room temperature (about 22 °C) in the dark, prior to fixing with formaldehyde. Cells that increased their length to 4–10 μm (vs. < 3 μm in soil at 4 months, when assessed in absence of yeast extract) were counted as nutrient responsive. At least 20 fields and/or 150 bacterial cells were studied per filter. When population levels were low, filters were examined until at least 10 elongated cells were found.

Colony counts of CHA0-Rif were obtained by spread plating onto solid King's B containing Rif100, which in the soil experiment contained also the antifungal compound cycloheximide (Fluka AG) at 190 μg mL^{-1}. Plates were incubated at 28 °C for 2 days before counting the colonies. Incubation of plates for additional days did not increase the number of colonies. When randomly chosen colonies were assessed by RAPD analysis (Keel *et al.*, 1996), results confirmed that they corresponded to strain CHA0-Rif. The background of resistance to Rif100 in the resident pseudomonads (and other bacteria growing on King's B), assessed by plating extracts from the uninoculated microcosms onto solid King's B containing Rif100, was less than 10^3 CFU g^{-1} dry soil (detection limit of 10^2 CFU g^{-1} dry soil), and colony morphology was different from that of strain CHA0-Rif. In addition, immunofluorescence microscopy showed that colonies resistant to Rif100 from uninoculated microcosms did not react with the CHA0-specific antiserum. At 2 weeks, colony counts (detection limit of 60 CFU g^{-1} dry soil) were also obtained for the indigenous fluorescent pseudomonads in noninoculated microcosms, using S1 medium (Gould *et al.*, 1985).

Statistics

The statistical design was completely randomized in both experiments. Treatments were duplicated in the *in vitro* experiment, which was run on two independent occasions with the same results. Each individual soil was studied in triplicate in the soil experiment. Cell numbers were log-transformed. Cell length of CHA0-Rif in soil was investigated in duplicate (100 cells per replicate), using two of three replicates of each soil. Cell length was Arcsine-transformed before analysis. Data were processed by one-factor (cell length) or two-factor (either pH *in vitro* × type of cell count, or soil × type of cell count) analysis of variance and (when appropriate) using Tukey's test ($P < 0.05$). Correlation analysis between soil properties and cell numbers or length was carried out using Pearson's coefficient ($P < 0.01$). All analyses were performed using R 2.10.1 software (http://www.r-project.org).

Results

Effect of pH stress on *P. protegens* CHA0-Rif *in vitro*

At 1 week, strain CHA0-Rif was found as culturable cells under *in vitro* conditions at pH 7 (Fig. 1). At pH 4, about 10^4 culturable cells were recovered vs. almost 10^8 total cells, among which about 10^7 nutrient-responsive cells. At pH 3, both total immunofluorescence counts and DVC of nutrient-responsive cells were comparable with the corresponding levels found at pH 4, whereas culturable cells were not found. Therefore, acidity resulted in the occurrence of non-cultured CHA0-Rif cells, a significant proportion of the latter (about 1 out of 1000 cells) being in a viable state.

Comparison of total cell counts and colony counts of *P. protegens* CHA0-Rif in 21 soils

In the five alkaline soils (pH > 7.4), total cell counts of *P. protegens* CHA0-Rif were *c.* 10^7 per g soil at 2 weeks and 10^6 at 2 months after inoculation (Fig. 2). Colony counts of the strain were equivalent to total cell counts in all cases, except that total cell counts were statistically higher in soils WIZ and DIE at 2 weeks and in soil HEL at 2 months (threefold differences).

In the five neutral soils (pH = 7.0 ± 0.3), total cell counts of strain CHA0-Rif amounted to *c.* 10^6 (soil WIT), 10^7 (soils ESC, SCH, SUG) or even 10^8 cells (soil RIB) per g soil at 2 weeks, vs. $10^{5–6}$ cells per g soil at 2 months (Fig. 2). As in alkaline soils, colony counts of strain CHA0-Rif were similar to total cell counts in all cases, except that total cell counts were statistically higher in soil RIB at 2 weeks and in soil SUG at 2 months (by up to a threefold difference).

In the five acidic soils (5.0 < pH < 6.5), total cell counts of strain CHA0-Rif were *c.* 10^6 (soil TAN), 10^7 (soil LAU), or between 10^6 and 10^7 cells (soils AUH, MOH, ZWE) per g soil at 2 weeks, and total cell counts exceeded colony counts in three soils, by a threefold (soil AUW) to a 10-fold difference (soil LAU; Fig. 2). At 2 months, total cell counts of strain CHA0-Rif were below quantification limit (i.e. 10^5 cells g^{-1} dry soil) in three soils (namely LAU, TAN, ZWE; where its colony counts were $10^{4–5}$ cells per g soil), whereas in both soils AUH and MOH, total cell counts of the pseudomonad (*c.* 10^6 cells per g soil) exceeded colony counts 10-folds.

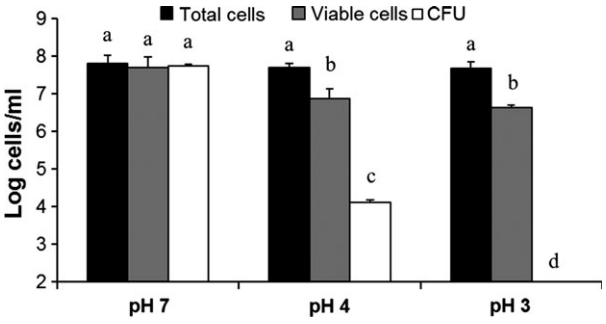

Fig. 1. Numbers of total cells, viable cells and culturable cells of *Pseudomonas protegens* CHA0-Rif at 1 week after incubation at pH 7, 4 or 3 under *in vitro* conditions (means ± standard deviations). Statistical relations ($P < 0.05$) between the six means are indicated with letters a–d (two-factor ANOVA followed by Tukey's tests).

In the six very acidic soils (pH < 5.0), total cell counts of strain CHA0-Rif were *c.* 10^7 (soil LAP) or between 10^7 and 10^8 cells (in the others) per g soil at 2 weeks, and about 10^5 (soil LAP), 10^6 (soils KRB, LAF, SIL) or 10^7 cells (soils KRA, SIG) per g soil at 2 months (Fig. 2). Total cell counts were statistically higher than colony counts in all cases, by a factor of 10 (soil LAP) to 300 (soil SIL) at 2 weeks and about 10 (soil LAP) to 1000 (soil LAF) at 2 months. This assessment was also carried out at 4 months, and it gave $10^{6–7}$ total cells of strain CHA0-Rif per g soil and from less than 10^2 (detection limit) to about 10^4 CFU of the pseudomonad per g soil (not shown).

Relation between soil properties and population size of *P. protegens* CHA0-Rif in soil

No correlation was found between soil pH and total cell counts of *P. protegens* CHA0-Rif, at 2 weeks or 2 months after inoculation (Fig. 3). However, there was a positive, significant correlation between pH and colony counts of the strain, both at 2 weeks ($r = 0.73$; $P = 2 \times 10^{-4}$) and 2 months ($r = 0.76$; $P = 7 \times 10^{-5}$).

There was no correlation at either sampling time (data not shown) between total cell counts or colony counts of strain CHA0-Rif (on one hand), and the percentage of major soil constituents (clay, silt, sand, organic matter), contents in N, P, K, S, Ca, Mg, Na, B, Fe, Cu, Mn or Zn, cationic exchange capacity, or soil water content at −0.03 MPa (on the other hand). There was no correlation either when considering soil texture classes (from sandy to clayey) or the main characteristics of the sampling sites in Table S1, that is, land use, altitude above sea level, site geology, type of soil horizon, depth of soil horizon and abundance of roots within the soil horizon sampled (data not shown).

Cell morphology of *P. protegens* CHA0-Rif in soil

The morphology of the cells of strain CHA0-Rif in soil was studied at 2 weeks after inoculation, by immunofluorescence microscopy of soil extracts. Cells from the

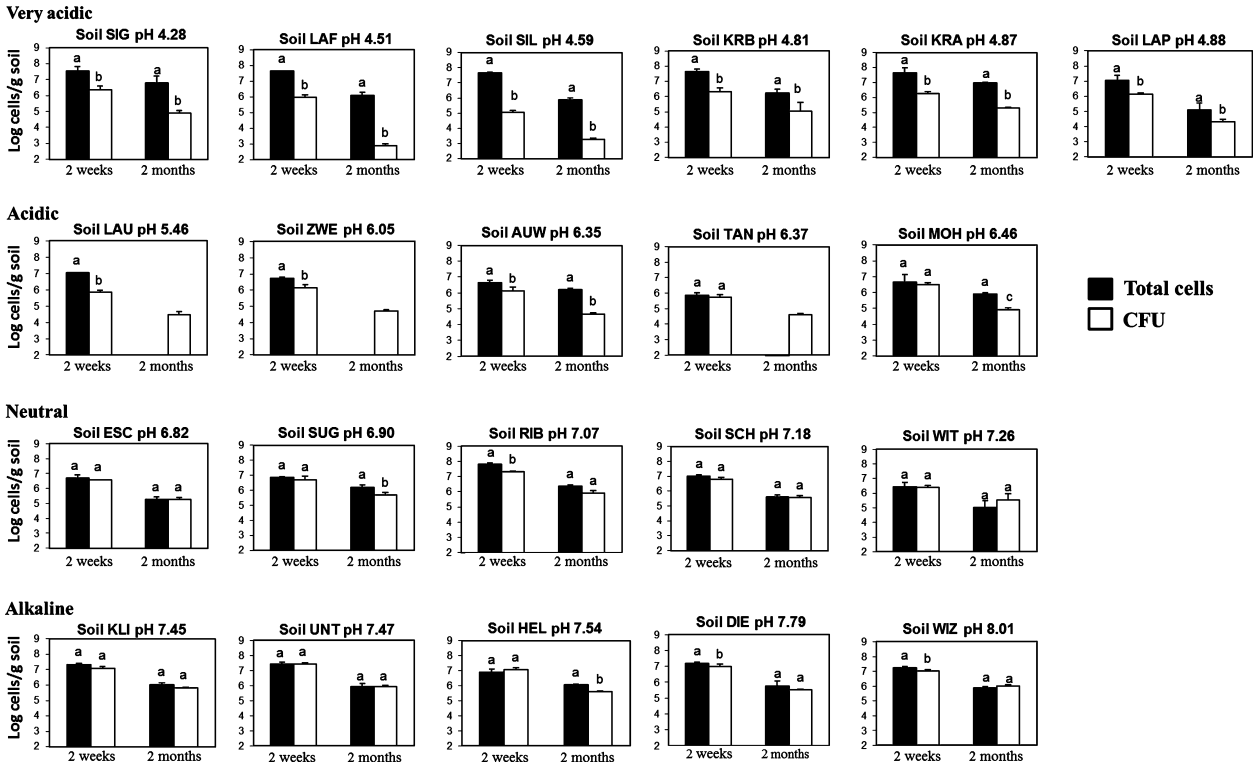

Fig. 2. Total cells and culturable cells of *Pseudomonas protegens* CHA0-Rif at 2 weeks and 2 months after inoculation in 21 natural bulk soils. Means ± standard deviation are shown. For each soil, the statistical relation ($P < 0.05$) at each sampling between total cell count and colony count is indicated using letters a and b.

inoculum were also investigated and results indicated that the length of the cells (rod-shaped) varied from 2 to 7 μm, with a mean of 3.5 μm (Fig. S1). Cell length patterns of CHA0-Rif in soil were similar to that in the inoculum for the 6 very acidic and the 5 acidic soils, that is, with a majority of cells longer than 3 μm, whereas in neutral and alkaline soils, almost all cells were smaller than 3 μm in length.

The relation between soil properties and cell length of CHA0-Rif was assessed based on the percentage of cells whose length in soil was 3 μm or less. As expected from the above, the correlation between this criterion and soil pH ($r = 0.86$; $P = 5 \times 10^{-7}$) was significant (Fig. 3). It was also significant with soil content in Ca ($r = 0.61$; $P = 0.0031$) or Na ($r = 0.56$; $P = 0.0082$). In contrast, there was no relation between the percentage of cells of CHA0-Rif 3 μm or less in length and any of the other soil properties or site characteristics (data not shown).

Relation between soil pH and number of indigenous culturable fluorescent pseudomonads

The indigenous culturable fluorescent pseudomonads were also investigated, to determine whether sensitivity to acidity was a trait specific to strain CHA0-Rif or shared also by indigenous pseudomonads. At 2 weeks, the number of indigenous culturable fluorescent pseudomonads in noninoculated bulk soil ranged from 3×10^2 (soil LAF) to 5×10^5 CFU (soil ESC) per g soil (Fig. S2). It was 10^5 CFU per g soil or higher for alkaline and neutral soils, between 5×10^3 and 4×10^5 CFU per g soil for acidic soils, and between 3×10^2 and 5×10^4 CFU per g soil for very acidic soils. In addition, the number of indigenous culturable fluorescent pseudomonads correlated with soil pH ($r = 0.71$; $P = 3 \times 10^{-4}$), but not with any other soil property (not shown). Similar results were obtained when counts were carried out on King's B instead of S1 medium (not shown).

Comparison of total cell counts, viable counts and colony counts of *P. protegens* CHA0-Rif at 4 months

A third sampling was performed at 4 months, in a subset of 11 soils, to verify the significance of noncultured CHA0-Rif cells using Kogure's method (by then all CHA0-Rif cells were < 3 μm and many even smaller). At that sampling, colony counts were similar to total cell counts in the alkaline soil, but fivefold lower in the

neutral soil, 40–130-fold lower in the three acidic soils and 2000-fold lower in the very acidic soil LAP (Fig. 4). Cells were below microscopy detection limit in the other very acidic soils. For the six soils in which microscopic tools could be used, the difference between Kogure's DVC and colony counts increased with acidity and became significant in the very acidic soil LAP (pH 4.88), where the number of nutrient-responsive cells was about 100 times higher than that of culturable cells.

Discussion

Pseudomonas cells may lose colony-forming ability upon exposure to certain abiotic stress and persist as nonculturable cells (Mascher *et al.*, 2000; Rezzonico *et al.*, 2003). In soil, this was shown for external stress factors related to soil management and/or climatic events, such as oxygen-deprived/reducing conditions above field

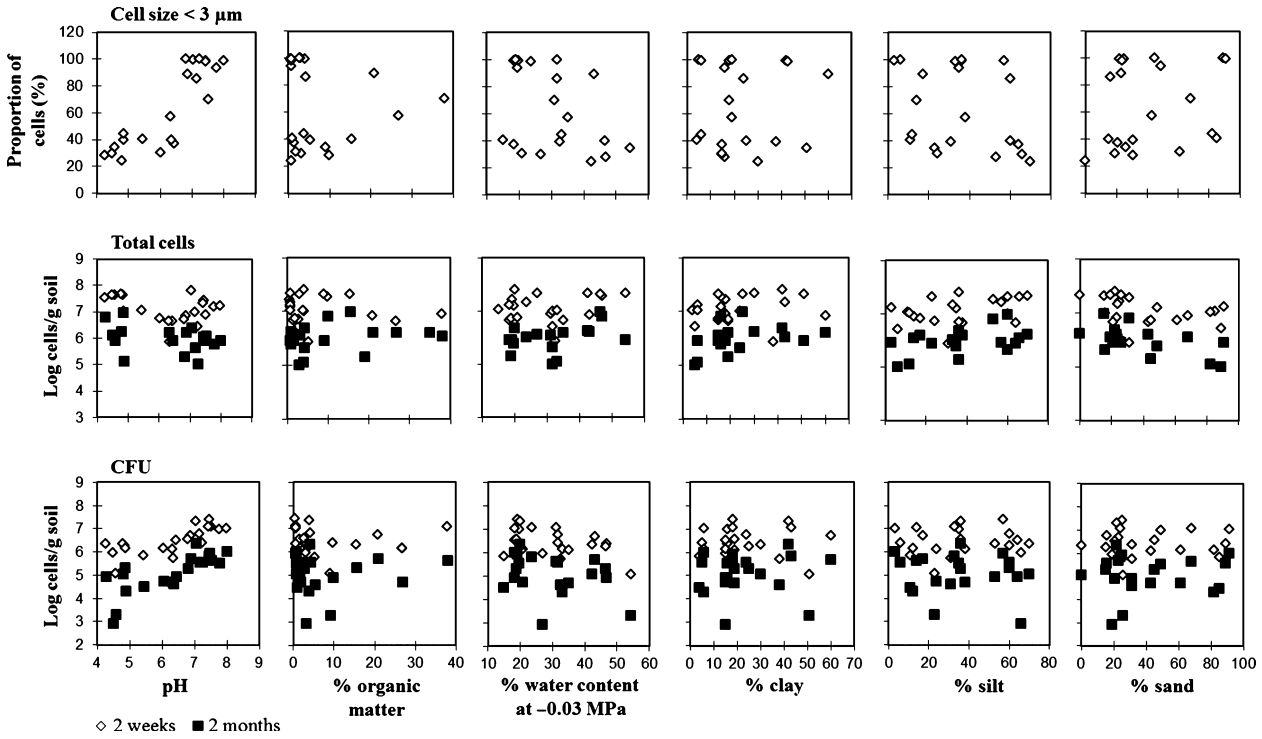

Fig. 3. Relation between (1) soil pH, organic matter content, water content at −0.03 MPa and contents in clay, silt, sand; and (2) the percentage of CHA0-Rif cells less than 3 µm in size (at 2 weeks), total cell count and colony count of *Pseudomonas protegens* CHA0-Rif (at 2 weeks and 2 months after inoculation) in 21 natural bulk soils.

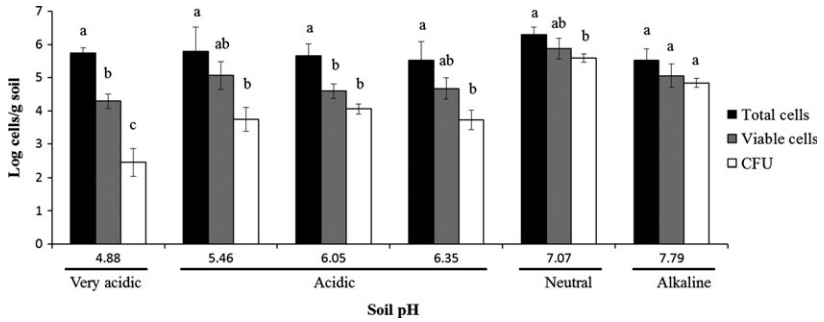

Fig. 4. Total cells, viable cells and culturable cells of *Pseudomonas protegens* CHA0-Rif (means ± standard deviation) at 4 months after inoculation in six natural bulk soils, that is, the very acidic soil LAP (pH 4.88), the three acidic soils LAU (pH 5.46), ZWE (pH 6.05), AUW (pH 6.35), the neutral soil RIB (pH 7.07) and the alkaline soil DIE (pH 7.79). Results for the five other very acidic soils are not shown as microscopic counts were below detection limit. For each soil, the statistical relation ($P < 0.05$) between total cell count, viable count and colony count is indicated using letters a–c.

plough pans (Mascher *et al.*, 2003; Troxler *et al.*, 2012), desiccation (Normander *et al.*, 1999) or freezing/thawing cycles (Troxler *et al.*, 1997b). In contrast, nothing was known on intrinsic soil properties conducive to *Pseudomonas* cell culturability loss, that is, soil characteristics that would render the soil stressful to pseudomonads even under favourable climatic incubation conditions. Following circumstantial observations *in vitro* (Rezzonico *et al.*, 2003) and in soil (Hase *et al.*, 2000), this work with *P. protegens* CHA0-Rif establishes for the first time the loss of colony-forming ability of a pseudomonad in soil under acidic conditions, to an extent that increases with soil acidity. On this basis, the usefulness of culturable bacteria including pseudomonads as sensitive metal stress indicators in soils (Brandt *et al.*, 2006) needs to be considered with caution if heavy metal contamination is accompanied by significant pH decrease.

Soil pH is a key feature influencing bacterial growth (Rousk *et al.*, 2009), activity and ecological role (Nicol *et al.*, 2008; Bakken *et al.*, 2012; Pereira e Silva *et al.*, 2012), as well as the structure (Nicol *et al.*, 2008; Lauber *et al.*, 2009) and size (Rousk *et al.*, 2010) of bacterial communities. However, not all bacterial taxa are sensitive to soil acidity. Various soil properties – but not pH – affected survival of *Azospirillum brasilense* strains Cd and Sp245 across 23 sterilized soils (Bashan *et al.*, 1995). Loss of colony-forming ability in *P. fluorescens* R2f took place in a loamy sand (pH 6.2) but not a silt loam (pH 7.5), suggesting a possible role for texture (van Overbeek *et al.*, 1995), but only two soils were assessed. In fact, the response of *P. protegens* CHA0-Rif to pH might be relevant for fluorescent *Pseudomonas* spp. at large, as the number of indigenous culturable fluorescent pseudomonads also diminished with more acidic pH values. Biocontrol activity of *P. fluorescens* 2–79 towards wheat take-all correlated with soil pH (Ownley *et al.*, 2003), but whether strain survival played a role was not investigated.

At 2 weeks, cell size distribution of *P. protegens* CHA0-Rif in the acidic and very acidic soils was comparable with the one in the inoculum, whereas in neutral and alkaline soils, most cells were smaller. As colony counts and total cell counts of strain CHA0-Rif were essentially equivalent at that sampling, in the latter soils, it indicates that a reduction in cell size was important for strain survival in neutral and alkaline soils. This is reminiscent of previous findings with this and other *Pseudomonas* strains in soil (van Overbeek *et al.*, 1995; Troxler *et al.*, 1997b). The fact that cell size distribution in the acidic and very acidic soils remained as in the inoculum is likely an indication that inoculant cells did not manage to adapt physiologically to adverse pH conditions in these soils. Indeed, CHA0-Rif cells at 4 months were smaller and about the same size in all soils, suggesting that larger inoculant cells had been eliminated by then.

The noncultured cells of *P. protegens* CHA0-Rif evidenced in this work were unlikely to be dead, as dead cells disappear rapidly in soil (Turpin *et al.*, 1993). Indeed, some of the noncultured cells of *P. protegens* CHA0-Rif were nutrient responsive in the very acidic soil LAP, where Kogure's DVC were about 100 times higher than colony counts. Similarly, a significant amount of CHA0-Rif cells were nutrient responsive when exposed to acidity *in vitro*, which did not take place when the strain was killed using H_2O_2 (Hase *et al.*, 2000). Kogure's DVC were lower than total cell counts when the inoculant was exposed to acidity, *in vitro* and in two of the four soils. This was perhaps due to the conservative cell size threshold used in Kogure's test (i.e. it is likely that many responsive cells did not reach this cell size threshold; Mascher *et al.*, 2003) and effects of the tetrasodium pyrophosphate soil extraction procedure on cell's nutrient responsiveness (although no negative effects were found with nonstressed cells). *Bac*Light staining showed that the majority of Kogure test-negative cells of *P. protegens* CHA0-Rif exposed to stress in soil were not dead (Mascher *et al.*, 2003), and these cells might be in a dormant state (Troxler *et al.*, 2012). It is important to note that noncultured cells of *P. protegens* CHA0 resulting from exposure to stress (including acidity) were not efficiently quantified by quantitative PCR (Rezzonico *et al.*, 2003).

In conclusion, the main soil composition property leading to cell culturability loss of *P. protegens* CHA0 in bulk soil was acidity, regardless of soil texture and organic matter content, and indeed, noncultured cells were also prevalent when the strain was exposed to acidity *in vitro*. As inoculant survival is crucial to ensure efficacy, these findings are important to better define the range of soil conditions suitable for *Pseudomonas* inoculations.

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Authors' contribution

F.M. and C.H. contributed equally to this work.

Statement

The sole intrinsic soil composition factor triggering cell culturability loss in the biocontrol agent *Pseudomonas protegens* CHA0 is soil acidity, as growth ability of the bacterium is retained only in nonacidic soils.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Cell length distribution of *Pseudomonas protegens* CHA0-Rif at 2 weeks after inoculation in 21 natural bulk soils (means ± standard deviation) and in the inoculum.
Fig. S2. Number of indigenous culturable fluorescent pseudomonads in 21 bulk soils (means ± standard deviation).
Table S1. Main properties of the field sites and horizons sampled.
Table S2. Main properties of the soil samples used in the study (according to Swiss soil classification system).
Table S3. Location of the soils studied in relation to the 1 : 25 000 soil map[a] of Switzerland.